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A transferrin-binding protein of *Trypanosoma brucei* is encoded by one of the genes in the variant surface glycoprotein gene expression site

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A transferrin-binding protein (TFBP) with an apparent molecular weight of 42 kd was purified from detergent-soluble membrane proteins of bloodstream forms of *Trypanosoma brucei*. The protein is not expressed in the insect-borne stage of the parasite's life-cycle. Purified TFBP can be converted from an amphiphilic to a hydrophilic form by cleavage with *T.brucei* glycosyl-phosphatidylinositol (GPI)-specific phospholipase C, demonstrating that the C-terminus is modified by a GPI-membrane anchor. The TFBP is encoded by an expression-site-associated gene [*ESAG 6* in the nomenclature of Pays *et al.* (1989) *Cell*, 57, 835–845] which is under the control of the promoter transcribing the expressed variant surface glycoprotein gene. The possible function of TFBP as a receptor for the uptake of transferrin in bloodstream forms is discussed.

Key words: expression site associated gene/glycosyl-phosphatidylinositol/transferrin-binding protein/*Trypanosoma brucei*/variant surface glycoprotein

Introduction

The mammalian-borne stage in the life-cycle of the parasitic protozoan, *Trypanosoma brucei*, is covered by a dense coat of the membrane-form variant surface glycoprotein (mfVSG), protecting invariant plasma membrane proteins from the components of the immune system (reviewed by Cross, 1990). VSGs are encoded by a family of 100–1000 different genes; one *T.brucei* strain may contain as many as 20 different expression sites (ESs), only one of which is usually active (see reviews by Borst, 1986; Pays and Steinert, 1988; Donelson, 1989; Cross, 1990). Each ES consists of a multi-cistronic transcription unit that contains, in addition to the *VSG* gene, at least seven expression-site associated genes (ESAGs) numbered sequentially from the gene closest to the *VSG* gene. Most of these genes code for putative membrane proteins with potential N-glycosylation sites and N-terminal signal sequences (Cully *et al.*, 1985, 1986; Kooter *et al.*, 1987, 1988; Gibbs and Cross, 1988; Alexandre *et al.*, 1988; Pays *et al.*, 1989; Son *et al.*, 1989; Hobbs and Boothroyd, 1990; Zomerdijk *et al.*, 1990). A number of hypothetical functions have been attributed to the ESAGs; they may code for minor plasma membrane proteins required for nutrient uptake in bloodstream forms (e.g. receptors or transporters) or for secreted proteins that inhibit the immune response of the host (Kooter *et al.*, 1987). More direct evidence indicates that *ESAG 1* encodes a membrane-associated glycoprotein (Cully *et al.*, 1986) and *ESAG 4* might encode a Ca^{2+}-dependent adenylate cyclase (Pays *et al.*, 1989; Rolin *et al.*, 1990). However, in no case has an *ESAG* gene product with a specific function been isolated.

Trypanosomes require serum lipoproteins (Coppens *et al.*, 1988; Black and Vandeweerd, 1989) and transferrin (D.Schell and N.Borowy, unpublished experiments) as growth factors, which are probably taken up by receptor-mediated endocytosis (Coppens *et al.*, 1987, 1988; Webster and Grab, 1988). In this report, we describe the purification of a transferrin-binding protein (TFBP), which might be involved in transferrin uptake by the parasite. We demonstrate that the TFBP is encoded by *ESAG 6* and belongs to the class of C-terminal modified membrane proteins containing a glycosyl-phosphatidylinositol (GPI) residue.

Results

Purification of a transferrin-binding membrane protein

Before embarking on the purification of a putative trypanosomal transferrin receptor, we reproduced the purification of the mammalian receptor from human placenta. Affinity chromatography on human transferrin coupled to Sepharose readily confirmed that the human receptor was released from its ligand by the chaotropic SCN^- ion (Fernandez-Pol and Klos, 1980; Rudolph and Regoeczi, 1987; Turkewitz *et al.*, 1988). A detergent extract from trypanosome membranes of variant 117a bloodstream forms did not yield a protein under the same conditions. However, the additional treatment of the column with 50 mM glycine, pH 2.0, led to the elution of a single protein with an apparent molecular weight of 42 kd (Figure 1a, lanes 2 and 3). In addition, small amounts of transferrin were released from the support (lanes 3–5). This elution pattern was faithfully reproduced in more than 10 independent experiments. About 1 μg protein was obtained from 10^{10} *T.brucei* cells (~60 mg total protein); this corresponds to 1000 molecules/cell.

The following experiments established that the 42 kd polypeptide is a membrane protein specific for bloodstream forms of *T.brucei*. Firstly, subjecting the transferrin column to the elution regimen without application of the detergent extract yielded only transferrin (Figure 1b, lane 2). Secondly, a mock purification on bovine serum albumin coupled to Sepharose yielded neither transferrin nor the 42 kd protein in the eluate (lane 3). Thirdly, the protein was not obtained when a detergent extract of rat blood cell membranes, which could contaminate trypanosomes, was passed over the transferrin affinity column (lane 4). Fourthly, trypanosome membranes were subjected to phase separation in Triton X-114 (Bordier, 1981), and both the detergent phase and

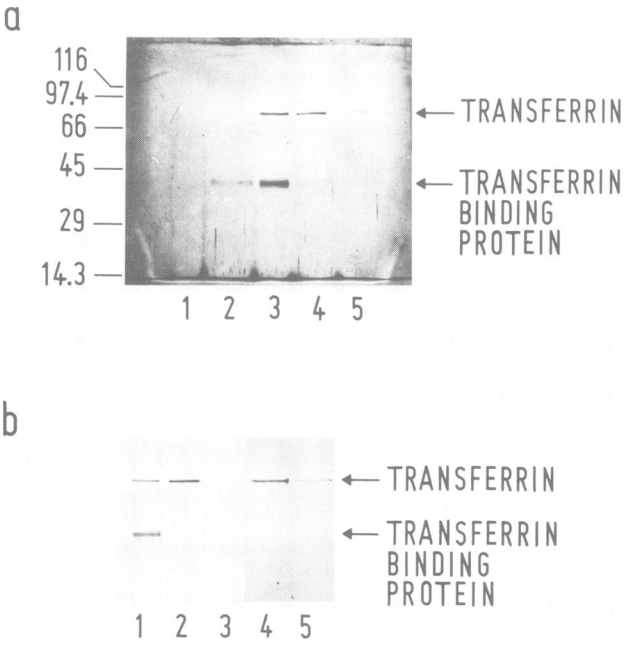

Fig. 1. Silver-stained polyacrylamide gels of fractions eluted from the transferrin affinity column. (**a**) Chromatography of a detergent extract from bloodstream forms. Lanes 1–5 refer to successive fractions collected after changing the elution conditions from 100 mM Tris, 500 mM NaSCN, 50 μg/ml Desferal, 0.2% Triton X-100, pH 8.0, to 50 mM glycine, 0.2% Triton X-100, pH 2.0. The 42 kd polypeptide is predominantly found in fraction 3 (lane 3). (**b**) Each lane refers to fraction 3 of the 50 mM glycine, 0.2% Triton X-100, pH 2.0, eluant using the following combinations of Sepharose 4B columns and extract; human transferrin and detergent extract of membranes from bloodstream forms [lane 1, independent purification of that shown in part (a)]; human transferrin and no detergent extract (lane 2); bovine serum albumin and detergent extract of bloodstream form membranes (lane 3); human transferrin and detergent extract of rat blood cells (lane 4); human transferrin and detergent extract of membranes from procyclic trypanosomes (lane 5).

the aqueous phase were subjected to affinity chromatography; the 42 kd polypeptide was entirely associated with the detergent phase (not shown). Finally, the purification protocol did not yield any transferrin-binding protein from membranes of procyclic trypanosomes (lane 5), however, the 42 kd polypeptide could be purified from membranes of bloodstream forms of another variant (221a), in which a different ES is active (see below).

Attempts to demonstrate the binding of transferrin to a non-reduced and unheated sample of the 42 kd polypeptide blotted from SDS–polyacrylamide gels to nitrocellulose filters were unsuccessful. Binding could be readily demonstrated when fractions eluted from the column were directly applied to nitrocellulose and probed with biotinylated human diferric transferrin and ExtrAvidin–alkaline phosphatase (Figure 2). While the fractions eluted with NaSCN were negative (dots 1–5), strong binding was observed to the third fraction of the acidic glycine eluate (dot 8), which corresponds to the fraction (Figure 1a, lane 3) containing the highest concentration of the 42 kd protein. Binding of biotinylated transferrin in the dot blot assay could be inhibited by a 40-fold excess of unlabelled transferrin (not shown). Finally, we were unable to detect specific binding of transferrin to Triton X-100 solubilized membrane proteins using this assay.

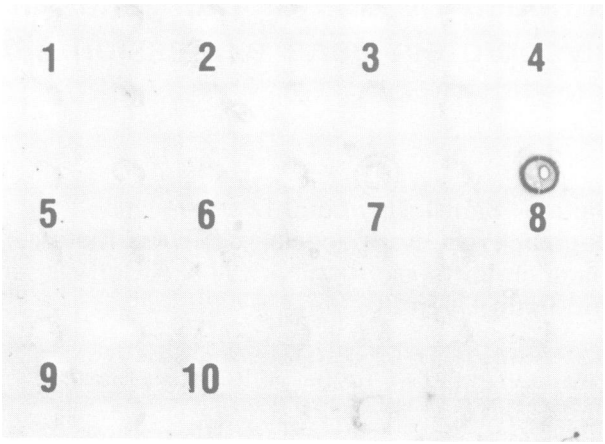

Fig. 2. Binding of transferrin to the purified 42 kd protein. An extract of bloodstream form membranes was subjected to affinity chromatography on a human transferrin affinity column as described in Materials and methods and documented in Figure 1. Aliquots (50 μl) of successive fractions eluted with 100 mM Tris, 500 mM NaSCN, 50 μg/ml Desferal, 0.2% Triton X-100, pH 8.0 (dots 1–5) or 50 mM glycine, 0.2% Triton X-100, pH 2.0 (dots 6–10) were applied to a nitrocellulose filter and probed with biotinylated human diferric transferrin and ExtrAvidin alkaline phosphatase. The positive reaction of dot no. 8 corresponds to fraction 3 (lane 3 in Figure 4), which shows the bulk amount of the 42 kd protein.

The transferrin-binding protein is encoded by an ESAG

The N-terminal amino acid sequence of the purified TFBP was determined by microsequencing (Figure 3b). Southern blot analysis of *Hin*dIII digested trypanosomal DNA was performed using an oligonucleotide (probe A) deduced from the peptide sequence (Figure 3b). This probe detected multiple fragments, suggesting that the gene for the TFBP belongs to a gene family. The fragment of 2.5 kb (Figure 4a) was isolated from a genomic library using oligonucleotide probe A (clone 7.2) and subsequently partially sequenced (Figure 3b). The deduced amino acid sequence from the 2.5 kb clone contained a signal sequence, which, after cleavage, predicted an amino terminus identical to that of TFBP. A homology search of databases with the transferrin-binding protein sequence revealed a high degree of sequence similarity to *ESAG 6* and *7* of *T.brucei* variant AnTat 1.3A (Figure 3b, Pays *et al.*, 1989). *ESAG 6* and *7* and the homologous gene family *X* (Kooter *et al.*, 1988) or *BS1* (Hobbs and Boothroyd, 1990) are found in different expression sites. This would predict that these genes are expressed in all *T.brucei* variants independent of the expression site that is used by the respective *VSG* gene. Therefore, we examined the presence of a mature transcript in *T.brucei* variants 1.8a, 1.8b, 117a, 221a and 118a, in which the active expression sites are situated on four or five different chromosomes (Van der Ploeg *et al.*, 1989; Gottesdiener *et al.*, 1990). The gene-internal probe B, encoding sequences homologous to the 5′ end of *ESAGs 6* and *7* (Figure 3a), hybridized to a message of ~1.7 kb in all variants analyzed (Figure 4b). This suggests that both genes code for transcripts of similar size as indicated by analysis of full-length cDNA clones for *ESAG 6* and *7* (Pays *et al.*, 1989). This probe also cross-hybridized to two larger RNAs of ~3.3 and

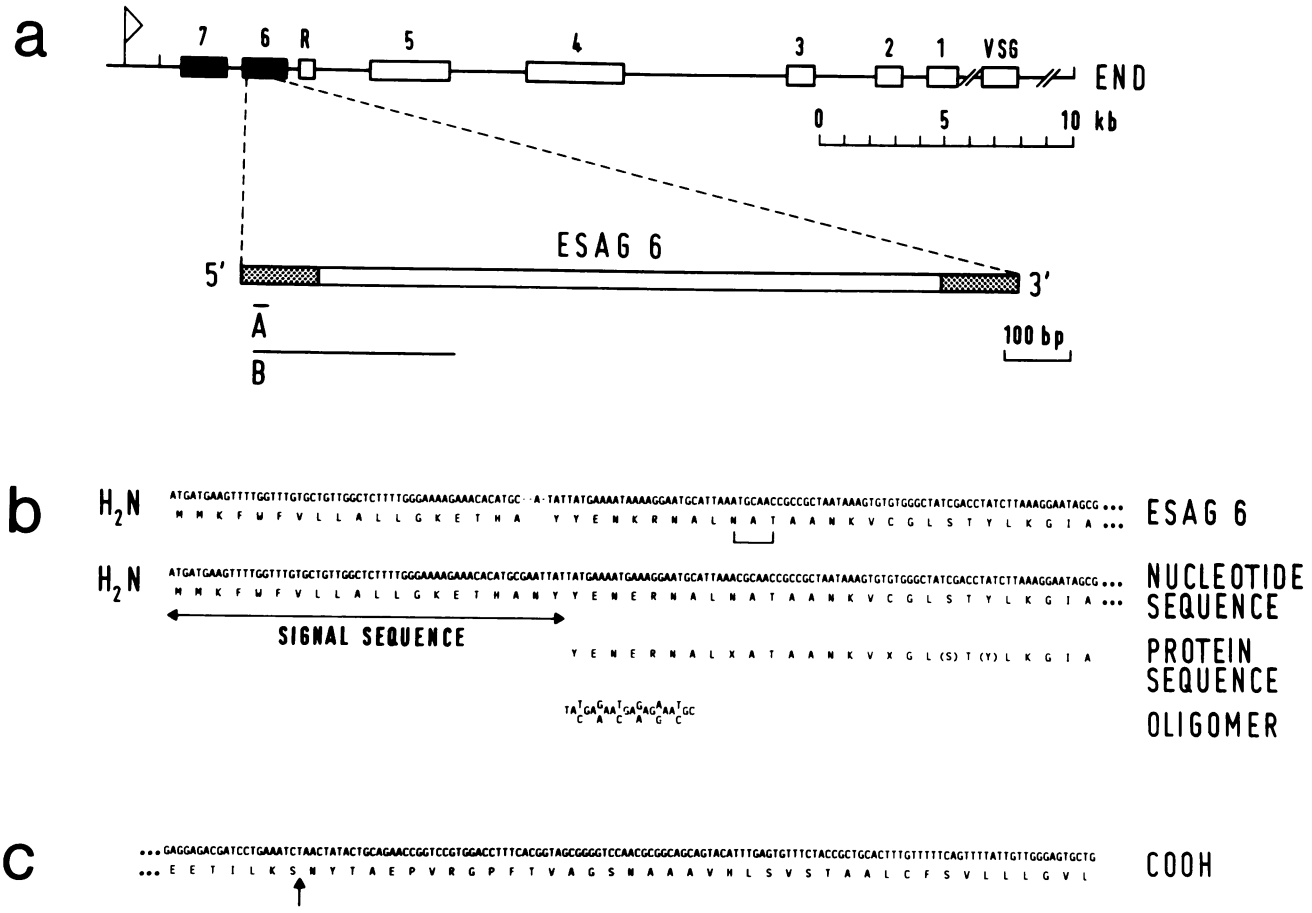

Fig. 3. Schematic representation of an active trypanosomal expression site (panel **a**). Active *VSG* genes always reside near the end of a chromosome (END). The expression site contains, in addition to the *VSG* gene (Kooter *et al.*, 1987; Pays *et al.*, 1989), expression-site associated genes (ESAGs; Cully *et al.*, 1985). Individual ESAGs are numbered 1–7. The flag denotes the position of the promoter directing transcription of the expression site. The box labelled 'R' corresponds to a RIME retroposon. The *ESAG 6* is shown below the map as a bar (Pays *et al.*, 1989); A and B indicate probes used. The hatched boxes represent the N- and C-terminal nucleotide and deduced amino acid sequences depicted in the first lines of panels **b** and **c**, respectively. A putative glycosylation signal (NAT) is underlined. The second line in panel b shows the nucleotide and deduced amino acid sequence of clone 7.2. The third line depicts the amino-terminal peptide sequence obtained by microsequence analysis of the purified transferrin-binding protein (X refers to unidentified residues, bracketed amino acids were only tentatively identified); this sequence was used to design an oligonucleotide probe (probe A) used in cloning the gene (line 4). The arrow in panel c indicates a possible site for the addition of the GPI-anchor.

4.0 kb. We do not know the origin of these RNAs. We could not detect an mRNA in Northern blots of total RNA of procyclic trypanosomes (Figure 4b). However, we did detect a signal if we enriched for poly(A)$^+$ RNAs. This signal was still very weak in comparison to that obtained with poly(A)$^+$ RNA of bloodstream trypanosomes (Figure 4c).

The transferrin-binding protein is modified by a GPI-anchor

The sequence of *ESAG 6* (Pays *et al.*, 1989) predicts a protein of 401 amino acids with an N-terminal signal sequence of 19 amino acids, a stretch of hydrophobic amino acids at the C-terminus characteristic for proteins modified post-translationally by a GPI-membrane anchor (Figure 3c, reviewed in Ferguson and Williams, 1988) and three potential glycosylation sites (one possibly removed by processing of the C-terminus, cf. Figure 3C). In contrast, the highly homologous *ESAG 7* is truncated at the 3′ end, encoding a putative protein of 340 amino acids (Pays *et al.*, 1989). Therefore, the size of TFBP suggested that it is encoded by *ESAG 6* rather than by *ESAG 7* and, moreover, that TFBP is modified by a GPI-anchor. Although TFBP was found in the detergent phase when membrane proteins were subjected to a Triton X-114 phase separation (see above), the purified TFBP partitioned into the aqueous phase (Figure 5, lanes 1 and 2) suggesting that during the standard purification in the presence of Triton X-100 TFBP was cleaved by the endogenous GPI-specific phospholipase C (GPI-PLC) (Cardoso de Almeida and Turner, 1983; Bülow and Overath, 1986; Fox *et al.*, 1986; Hereld *et al.*, 1986). A modified purification procedure (preparation of the membrane extract using the detergent Zwittergent 3-12 and the sulfhydryl reagent *p*-chloromercuribenzene sulfonate, see Materials and methods), which ensured the inhibition of the phospholipase, predominantly yielded an amphiphilic form of TFBP that could be converted to a hydrophilic form by the treatment with purified *T.brucei* GPI-PLC (Figure 5, lanes 3–6).

Cleavage of GPI-anchored proteins by GPI-PLC produces diacylglycerol and peptidyl-glycosylinositolcyclophosphate, which is specifically recognized by so called cross-reacting antibodies (Zamze *et al.*, 1988; reviews by Ferguson and Williams, 1988; and Low, 1989). TFBP purified by the standard procedure could be readily detected by such antibodies and this reaction was not enhanced by GPI-PLC treatment (Figure 6, lanes 1 and 2). In contrast, TFBP purified by the modified procedure reacted only weakly with the antibody

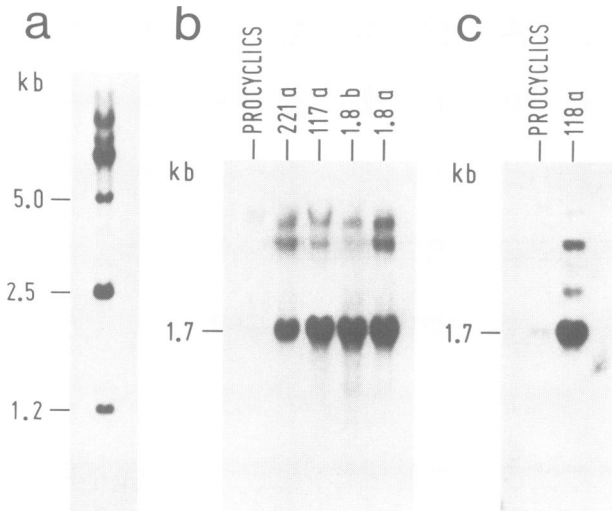

Fig. 4. Identification and Northern analysis of the gene encoding the transferrin-binding protein of *T.brucei*. (**a**) 4 μg of DNA from variant 118a were digested with *Hin*dIII, size-fractionated on a 0.7% agarose gel, and transferred to nitrocellulose. The blot was hybridized with probe A (Figure 3a) and washed to a final stringency of 3 × SSC at 55°C (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0). (**b**) 20 μg total RNA of procyclic trypanosomes and bloodstream forms of variants 221a, 117a, 1.8b and 1.8a were size-fractionated on a 1% formaldehyde agarose gel and transferred to a Biodyne B nylon membrane. The blot was hybridized with the nick-translated gene-internal probe B (homologous to nucleotides 0–310 of ESAG 6, see Figure 3a) and washed to a final stringency of 0.1 × SSC at 65°C. (**c**) 6 μg of poly(A)$^+$ RNA of procyclic trypanosomes and bloodstream forms of variant 118a were separated and hybridized as described in panel b. The insert of clone 7.2 was used as probe in this hybridization.

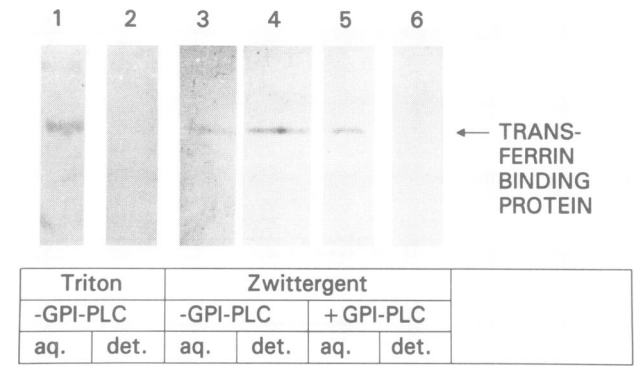

Fig. 5. Treatment of transferrin-binding protein with GPI-specific phospholipase C. The figure shows silver-stained SDS–polyacrylamide gels. TFBP purified by the standard protocol using the detergent Triton X-100 was subjected to a Triton X-114 phase separation. The protein is associated with the aqueous (lane 1) rather than the detergent phase (lane 2). TFBP was purified by a modified procedure using PCMBS and the detergent Zwittergent 3-12 (see Materials and methods) in order to inhibit the endogenous phospholipase C. Elution of TFBP was performed in the presence of 0.2% Triton X-114. Upon phase separation (lane 3, aqueous phase; lane 4, detergent phase) TFBP is almost exclusively found in the detergent phase. After subjecting this preparation to treatment with purified *T.brucei* phospholipase C (in the presence of 50 mM dithioerythritol and 0.5 mM EDTA), the protein was converted from an amphiphilic form to a hydrophilic form (lane 5, aqueous phase, lane 6, detergent phase).

before the lipase treatment but strongly thereafter (lanes 3 and 4). Upon Triton X-114 phase separation of the GPI-PLC-treated material, the immunoreactive protein was predominantly associated with the aqueous phase (lanes 5 and 6).

Discussion

The N-terminal sequence of TFBP isolated from variant 117a (Figure 3b) is identical to the corresponding *ESAG 6* or *ESAG 7* sequences derived from clones of the active ES from variant AnTat 1.3A (Pays *et al.*, 1989) or the sequence from genomic clones of the *BS1* gene of variant 117a (Hobbs and Boothroyd, 1989), with the exception of a $Glu^4 \rightarrow Lys^4$ change. The sequence reported for a cDNA clone from variant 117a does not cover the 5′ end of the gene (Hobbs and Boothroyd, 1989). A Glu residue at position four is predicted from the genomic clone isolated in this study (Figure 3b); we do not know whether this clone is derived from the active or one of the many inactive ESs. These data lead to the conclusion that TFBP is encoded by *ESAG 6* or *7*. The relative molecular weight of 42 000 for TFBP as determined by gel electrophoresis is consistent with a mature *ESAG 6* product (derived from a 401-residue precursor, cf. Pays *et al.*, 1989) that lacks the N-terminal signal sequence and the hydrophobic C-terminus but which is modified by a GPI-anchor and Asn-linked carbohydrate chains. At least the Asn9 residue (Figure 3b) of the mature polypeptide is likely to be glycosylated because this amino acid could not be identified by protein sequencing. However, a highly N-

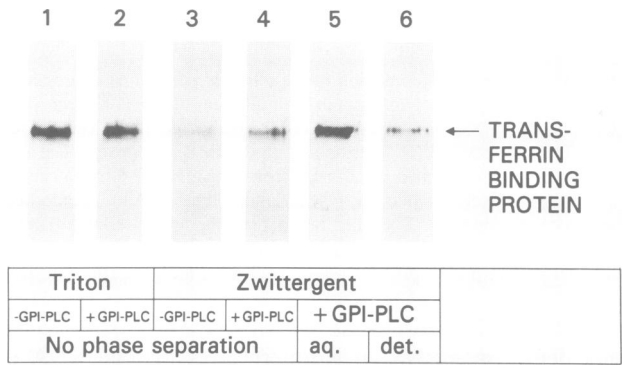

Fig. 6. Reaction of purified TFBP with cross-reacting antibodies. The figure shows immunoblots probed with cross-reacting antibodies and goat anti-rabbit IgG coupled to alkaline phosphatase and developed using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Lanes 1 and 2: TFBP purified by the standard protocol in Triton X-100. The 42 kd protein reacts with antibodies (lane 1); treatment with GPI-PLC does not enhance the reaction (lane 2). Lanes 3–6: TFBP purified in the presence of Zwittergent 3-12. The 42 kd protein weakly reacts with antibodies (lane 3); GPI-PLC treatment strongly enhances the reaction (lane 4). If the protein is subjected to a Triton X-114 phase separation after GPI-PLC treatment, the immunoreactive material is mainly associated with the aqueous phase (compare lanes 5 and 6).

glycosylated *ESAG 7* product (321 amino acid residues after cleavage of the N-terminal signal sequence) could have a similar electrophoretic mobility. The decision for *ESAG 6* rather than *ESAG 7* is based on the observation that, as predicted from the nucleotide sequence, TFBP is modified by a C-terminal GPI-anchor that is sensitive to cleavage by the *T.brucei* phospholipase C (Figure 5) and reacts after enzymatic cleavage with antibodies against the cross-reacting determinant (Figure 6).

TFBP is only found in bloodstream and not in procyclic forms, consistent with the well established stage-specific expression of the multi-cistronic VSG transcription unit. This result suggests that TFBP may only be required in bloodstream forms. So far, TFBP has been isolated from only two variant clones (117a and 221a), in which different expression sites are active. Since the amino acid sequences predicted from *ESAG 6* genes of different ESs are highly homologous but not identical, it is important to establish the presence of a transferrin-binding activity in other variant clones. Probe B (Figure 3a) readily detects a transcript derived from different expression sites (Figure 4b). However, since the mRNAs transcribed from *ESAG*s 6 and 7 are of the same size (Pays *et al.*, 1989) the 1.7 kb band detected comprises the sum of both of these transcripts. Further experiments are required in order to differentiate between the level of these transcripts.

While the binding of transferrin to the purified TFBP could be readily demonstrated (Figure 2), no specific reaction was detectable in dot blots of membrane proteins solubilized in the detergent Triton X-100. Moreover, a sequence-specific antiserum against synthetic peptides corresponding to the N-terminus of TFBP strongly reacted with the purified 42 kd protein in immunoblots but neither this nor any other polypeptide, i.e. a candidate *ESAG 7* product, could be detected in immunoblots of cells solubilized in SDS (D.Schell, A.Beck-Sickinger and G.Jung, unpublished experiments). These observations suggest that TFBP is indeed present in a very low copy number and that an estimate in the order of one or a few thousand molecules/cell is not unreasonable. We do not believe that a major fraction of TFBP is lost by phospholipase cleavage during membrane preparation because the protein could not be affinity-purified from the supernatant and because most of the mfVSG was retained in the particulate fraction, provided the procedure was carried out in the cold. However, we cannot exclude losses on the transferrin-affinity column.

Does the binding protein characterized in this report function as a receptor for the uptake of transferrin in trypanosomes? In contrast to the mammalian transferrin receptor (a disulfide-linked homodimer of 95 kd monomers composed of extracellular, membrane-spanning and cytoplasmic domains, see reviews by Dautry-Varsat and Lodish, 1984; May and Cuatrecasas, 1985; Thorstenson and Romslo, 1990), TFBP is a GPI-anchored protein only about half as large and not dimerized by disulphide bridges (D.Schell, unpublished observations). The amino acid sequences of the two proteins are not homologous and a polyvalent antiserum against the human receptor does not react with TFBP in immunoblots (D.Preis and D.Schell, unpublished observations). Moreover, the binding of the two proteins to transferrin appears to be different because they are not released under the same conditions from the affinity matrix.

After synthesis and modification by a GPI-anchor, TFBP is probably transported to the flagellar pocket of the parasite by the conventional route for glycoproteins (Duszenko *et al.*, 1988). The flagellar pocket is formed by an invagination of the plasma membrane at the arising flagellum (see Balber, 1990, for review); the membrane lining the pocket is the only site for excocytosis and endocytosis in bloodstream trypanosomes (Brown *et al.*, 1965; Langreth and Balber, 1975; Fairlamb and Bowman, 1980; Coppens *et al.*, 1987; Webster and Grab, 1988; Webster, 1989). Once inserted in the pocket membrane, TFBP could be loaded with transferrin and the complex could be rapidly internalized, thereby preventing distribution of the protein over the cell surface. Therefore, as a privileged, basically extracellular compartment, the pocket could protect the parasite in the blood and tissues of the host from antibody or cell-mediated cytotoxic mechanisms directed against conserved and functionally important proteins.

Materials and methods

Trypanosomes

Bloodstream forms of the cloned variant antigen types of *T.brucei* 427 (Cross, 1975) were used: MITat 1.4 (117a) (Cross, 1975; Bernards *et al.*, 1981); MITat 1.2 (221a) (Cross, 1975; Bernards *et al.*, 1984); MITat 1.5 (118a) (Cross, 1975; Michels *et al.* 1983) and 1.8a and 1.8b (Michels *et al.*, 1984). Procyclic trypanosomes derived from variant MITat 1.4 were grown as described by Brun and Schönenberger (1979).

Reagents

Human diferric transferrin (Sigma Chemie GmbH, Deisenhofen) was purified by anion exchange and gel permeation column chromatography (MonoQ HR 5/5, Superose 12 HR10/30; Pharmacia-LKB GmbH, Freiburg). The GPI-specific phospholipase C was purified by the method of Bülow and Overath (1986). Antibody against the cross-reacting determinant of the soluble form of VSG was kindly provided by R.Bülow.

Human diferric transferrin (10 mg in 500 μl 100 mM Na-carbonate buffer, pH 9.5) was reacted in a molar ratio of 1:5 with biotinyl-ε-amidocaproic acid *N*-hydroxysuccinimide ester (258 μg in 25.8 μl dimethylsulfoxide, Sigma) for 4 h at room temperature. After addition of 500 μl 100 mM Tris, pH 8.0 (30 min incubation), biotinylated transferrin was extensively dialysed against PBS at 4°C.

Proteins were coupled to activated CH-Sepharose 4B at a ratio of 10 mg/ml swollen gel according to the suppliers instructions (Pharmacia−LKB).

Purification of the transferrin-binding protein

T.brucei variant clone 117 was isolated from the blood of infected rats by diethylaminoethyl−cellulose chromatography (Lanham and Godfrey, 1970). Cells (5×10^{10}) pelleted by centrifugation were lysed by resuspension in 50 ml cold 5 mM HEPES, 50 μg/ml Desferal (Ciba-Geigy, Basel, Switzerland), 0.1 mM tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mM EDTA, pH 7.0, on ice. The lysate was sonicated for 1−2 min and subsequently centrifuged at 5°C for 60 min at 114 000 *g*. The membrane pellet was washed once in 50 ml of the same buffer and then taken up in 50 ml PBS, 2% Triton X-100, 0.1 mM TLCK, pH 7.2, using a Potter-type homogenizer. After another sonication (2 min), the suspension was stirred for 60 min on ice and then centrifuged as described above. The supernatant was used for the subsequent purification.

The purification was performed at 5°C. A column (530 μl bed volume) was packed with human diferric transferrin coupled to CH-Sepharose. The following solutions were applied to the column at a rate of 0.5 ml/min (0.2 ml/min for the detergent extract): 20 ml PBS, 0.2% Triton X-100; 5 ml 100 mM NH_4HCO_3, 1 mg/ml $Fe^{3+}NH_4$-citrate (Serva, Heidelberg), 0.2% Triton X-100 (Van Driel *et al.*, 1984); 10 ml PBS, 0.2% Triton X-100; 50 ml detergent extract; 20 ml PBS, 0.2% Triton X-100; 10 ml 100 mM citrate, 50 μg/ml Desferal, 0.2% Triton X-100, pH 5.0 (Anderson *et al.* 1986); 10 ml 100 mM Tris, 50 μg/ml Desferal, 0.2% Triton X-100, pH 8.0; 10 ml 100 mM Tris, 500 mM NaSCN (Rudolph and Regoeczi, 1987; Turkewitz *et al.*, 1988), 50 μg/ml Desferal, 0.2% Triton X-100, pH 8.0; 10 ml 50 mM glycine, 0.2% Triton X-100, pH 2.0. The eluate of the last two buffers was collected in 1 ml fractions. The glycine fractions were neutralized by collecting into tubes containing 1 M Tris, pH 8.0. This highly standardized elution protocol was used for all control purifications described in Figure 1b. The attempt to purify a transferrin-binding protein from procyclic trypanosomes was performed with 4×10^{10} cells exactly as for bloodstream forms.

Purification of the GPI-anchored version of TFBP was performed by a modification of a procedure described for mfVSG (Schell and Overath, 1990). Bloodstream forms were lysed in 5 mM HEPES, 0.1 mM TLCK, 0.1 mM EDTA, 10 mM *p*-chloromercuribenzene sulfonate (PCMBS), pH 7.0. The membrane fraction was extracted using PBS, 0.1 mM TLCK, 10 mM PCMBS, 2% Zwittergent 3-12 (Calbiochem, Frankfurt), pH 7.0. The purification was performed as described above except that all buffers used before application of the detergent extract to the column contained 0.7% Zwittergent while the buffers used thereafter contained 0.2% Triton X-114 or 0.2% Triton X-100.

Assay for transferrin binding activity

The protein in 50 μl aliquots of the column fractions was immobilized on nitrocellulose (0.22 μm pore size, Schleicher and Schüll, Dassel). The following steps were then performed at 20°C with slow agitation on a shaker: 5% skim milk powder in PBS (1 h); 50 ml PBS (3 times, 5 min); 1 mg biotinylated transferrin in 40 ml PBS (1 h); 50 ml PBS (3 times, 5 min); 20 ml ExtrAvidin–alkaline phosphatase (Sigma, 1:4000 in PBS, 2 h), 50 ml PBS (3 times, 5 min); 50 ml 50 mM $NaHCO_3$, 2 mM $MgCl_2$, pH 9.5 (5 min); 40 ml $NaHCO_3$, 2 mM $MgCl_2$, pH 9.5, containing 2 mg nitro-blue tetrazolium and 1 mg 5-bromo-4-chloro-3-indolyl phosphate (~10 min).

Amino acid sequence analysis

TFBP was applied to an 8% SDS–polyacrylamide gel and blotted onto siliconized glass fibre (Eckerskorn *et al.*, 1988), stained with Coomassie blue, excised and sequenced using an Applied Biosystems 477A gas phase sequencer, equipped with an on-line 120 A PTH amino acid analysis system.

SDS–PAGE and protein determination

Slab gels were run with 4.5% acrylamide in the stacking gel and 10% acrylamide in the separation gel (Laemmli, 1970). Gels were stained with Coomassie brilliant blue or silver (Heukeskoven and Dernick, 1985). For determination of the yield of the transferrin-binding protein, an aliquot was run on a gel together with standard amounts of bovine serum albumin (0.03–1.0 μg). The silver stained bands were evaluated by laser densitometry.

Molecular biological techniques

The procedures for DNA and RNA isolation, and Southern and Northern blot hybridization were as described (Maniatis *et al.*, 1982; Evers *et al.*, 1989). Genomic DNA from the *T.brucei* variant 118a was digested with *Hin*dIII and size selected fragments of 2–2.6 kb were cloned in pEMBL plasmids (Dente *et al.*, 1983). Recombinant plasmids were screened according to standard protocols (Maniatis *et al.*, 1982), with a degenerate oligonucleotide probe (TA(T/C) GA(G/A) AA(T/C) GA(G/A) AG(A/G) AA(C/T)GC), deduced from the N-terminal amino acid sequence of the transferrin-binding protein (see Figure 3b). The initial identification and sequencing of clone 7.2 (2.5 kb *Hin*dIII fragment) were performed on double stranded plasmid DNA with the degenerate oligomer as primer, using the Amersham Microtiterplate sequencing system. Clone 7.2 contains a 1.6 kb *Pst*I fragment, which was completely sequenced. These sequences were determined from single stranded templates (Dente *et al.*, 1983) by the chain termination method (Sanger *et al.*, 1980) using 7-deaza dGTP as a substitute for dGTP in all reactions.

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